Protozoan Infections

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I. INTRODUCTION

Numerous genera of protozoa infect humans. These multiform organisms are responsible for remarkably diverse infections. Route of parasite entry, location of infection, clinical manifestations, and antiparasitic host defences vary tremendously. It is impossible to describe in detail the contributions of natural immunity to resistance to infection or recovery

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from infection with each of this complex group of organisms. In this chapter, we emphasise interactions of *Leishmania* with non-specific defences, and illustrate principles of these interactions using other parasites.

II. CLINICAL DESCRIPTION AND IMMUNOPATHOPHYSIOLOGY

Leishmania species are important aetiologic agents of human disease in tropical and subtropical regions. Small animals serve as reservoirs of infection; the organism is transmitted between animals and humans by inoculation of the promastigote form of the parasite into skin by sandfly vectors as they ingest a blood meal. Cutaneous leishmaniasis, caused by *L. major* or *L. tropica* in the Old World and *L. mexicana* or *L. braziliensis* in the New, consists of skin ulcers at the sites of sandfly bites. Lesions begin several weeks after inoculation of the parasite, and may persist for many months before they resolve without antimicrobial therapy. Systemic manifestations are usually absent, but metastasis of viable parasites to regional lymph nodes has been repeatedly demonstrated. A more severe form of disease, mucocutaneous leishmaniasis, initially resembles cutaneous disease. After apparent spontaneous healing, however, lesions reappear in the nasal and oral mucosa and other structures of the face. Disease progresses to enlarging local lesions with mutilating destruction of cartilage and soft tissue. Mucocutaneous leishmaniasis eventually causes death by respiratory compromise, superinfection, or inanition. It is possible that characteristics of the parasite rather than of the host most forcefully determine whether cutaneous or mucocutaneous disease occurs as a consequence of infection: disease expression differs depending on the species of infecting organism. Moreover, skin responses to leishmanial antigens and lymphocyte mitogenic responses to lectins or antigens are similar in both conditions (Castes *et al.*, 1983). Delayed cutaneous hypersensitivity to leishmanin, a preparation of formalinised promastigotes, is generally present concurrently with the lesions, and persists during and after convalescence.

In contrast to patients with cutaneous or mucocutaneous disease, patients with diffuse cutaneous leishmaniasis do not manifest delayed hypersensitivity to intradermal injection of leishmanin; nor do their lymphocytes divide in response to stimulation with leishmanial antigen in vitro (Castes *et al.*, 1983). These patients have numerous, widely distributed skin lesions that contain abundant parasites. In many ways, this disease form resembles lepromatous leprosy. It is thought that failure to mount an appropriate cellular immune response underlies the heavy parasite burden of diffuse cutaneous disease (Convit *et al.*, 1972). Whether this failure is attributable to characteristics of the leishmanial

species or of the host is not yet known. *L. mexicana amazonensis,* which causes diffuse cutaneous leishmaniasis, also causes localised cutaneous disease. The relative contributions of host and parasite factors to disease manifestations are particularly difficult to determine in this instance because taxonomy of *Leishmania* is still based largely on clinical grounds. As yet, there is little general agreement on classification of these organisms by the numerous biochemical and molecular approaches that have been suggested (Lainson, 1983).

In contrast to cutaneous leishmaniasis, visceral leishmaniasis is caused by only three species of *Leishmania; L. donovani* or *L. donovani infantum* in the Old World and *L. chagasi* in the New (Wirth *et al.*, 1986). A nodular lesion develops at the site of parasite injection by sandflies. The parasite then disseminates to infect macrophages throughout the reticuloendothelial system. Macrophages in spleen, liver and bone marrow may be heavily parasitised. Clinically uninvolved skin may also contain large numbers of infected cells. As in diffuse cutaneous disease, the leishmanin skin test in visceral leishmaniasis is negative initially, but may become positive when patients recover from disease (Rees *et al.*, 1981). Similarly, patient lymphocytes stimulated with leishmanial antigens in vitro respond poorly during acute infection. Response improves, however, with treatment (Carvalho *et al.*, 1981; Haldar *et al.*, 1983).

The diagnosis of leishmanial infection is confirmed by demonstration of the organism in tissues, either by biopsy, culture, or recently by hybridisation with radiolabelled DNA probes (Wirth and McMahon-Pratt, 1982). All forms of leishmaniasis respond to treatment with antimony compounds. Mucocutaneous and diffuse cutaneous disease, however, may relapse or respond poorly to antimicrobial therapy.

Organisms closely related to *Leishmania* include *Trypanosoma cruzi,* which causes American trypanosomiasis (Chagas' disease) and *T. gambiense* and *T. rhodesiense,* which cause African sleeping sickness. The trypomastigote form of *T. cruzi* is deposited on the skin by infected reduviid bugs when they defecate after eating a blood meal. Parasites enter the circulation through breaks in the skin and disseminate widely. They enter macrophages or smooth or cardiac muscle cells, where they transform into amastigotes. The amastigotes multiply, burst their host cell, and circulate as trypomastigotes. In this acute phase of the disease, parasites are easily demonstrable in blood and other tissues. Symptoms are those of acute infection with local findings at the site of inoculation. Acute myocarditis or meningoencephalitis may occur during the acute phase. In survivors, a quiescent period ensues. Years later, chronic disease (cardiomyopathy or megaoesophagus or megacolon) may develop. Few parasites are evident at this time. Late disease may be a consequence of autoantibodies directed against cardiac muscle, but

conclusive evidence for this hypothesis is lacking. A number of studies indicate that cellular immunity plays a role in destruction of *T. cruzi*, presumably during early stages of disease (Kierszenbaum, 1979). In vitro, lymphokines induce intracellular killing of *T. cruzi* by macrophages. These findings suggest that lymphocyte–macrophage co-operation provides the basis for control of acute Chagas' disease. Whether continued lymphocyte-mediated cytotoxic activity plays a role in late-stage disease is presently unknown.

In contrast to Chagas' disease, African trypanosomiasis is overwhelmingly characterised by interactions of parasites with specific antibody. After a period of systemic complaints, the disease is manifest as progressive central nervous system impairment, characterised initially by inattention, and finally by stupor, coma and death in untreated patients. African trypanosomiasis is immunologically fascinating because the organisms continuously change their surface antigens in response to selective pressure by host antibody. As a consequence of these changes, successive waves of parasitaemia with different antigenic types of trypanosomes occur until the host dies. Natural immunity plays little role in the unsuccessful defence against these organisms. Although phagocytic cells are primarily responsible for destruction of the parasite, they act fundamentally in concert with specific, opsonising antibody. The host's inability to match parasite antigenic variation and the immunological consequences of antigen–antibody interactions, especially perivascular mononuclear infiltration and vasculitis, eventually lead to the patient's demise.

Toxoplasma gondii infects a wide range of animals, including humans. The parasite undergoes sexual reproduction only in felines, the definitive hosts. Felines are required to maintain the life cycle in nature, since incidental hosts do not excrete the parasite in their faeces. Humans, an incidental host, acquire toxoplasmosis transplacentally, by ingestion of oocysts excreted by felines, or by ingestion of tissue cysts from undercooked meat of infected animals, especially sheep and pigs. *Toxoplasma* infects one-third to one-half of normal humans by age 40, but causes recognisable disease in only a few of these individuals. After ingestion, the parasite excysts in the human digestive tract. Trophozoites disseminate to virtually all organs, where they parasitise a wide range of cell types, including skeletal and cardiac muscle, fibroblasts, and macrophages. In normal individuals, the most common symptoms of primary infection include fever and generalised or localised (usually cervical) lymphadenopathy. This phase generally subsides without treatment as replication of trophozoites is controlled and encystment occurs. The

organism rarely causes recurrent disease. In individuals who have been immunosuppressed by haemopoietic malignancy, cytotoxic chemotherapy, or other acquired cellular immunodeficiency conditions, (including acquired immunodeficiency syndrome. AIDS), the organisms may excyst, recommence replication, and cause illness. Encephalitis is the most common manifestation of acute toxoplasmosis in such individuals (Ruskin and Remington, 1976). The most significant consequence of *Toxoplasma* infection may lie in its effect on the fetus of infected mothers. Of mothers who acquire primary infection with *Toxoplasma* in the first trimester, 30–50 per cent deliver an obviously affected infant. Congenital infection is associated with hydrocephalus, chorioretinitis, hepatosplenomegaly, and rash. In survivors, mental retardation, deafness, and cardiac malformations may be noted. Chorioretinitis may also appear in adulthood as a sequela of congenital infection.

Although control of *Toxoplasma* infection clearly requires participation of components of cell-mediated immunity (Frenkel, 1967), the mechanistic basis for inhibition of parasite replication remains unknown. Present in vitro models are most germane to control of the initial infectious episode. Most published work examines the ability of lymphokine-treated macrophages to destroy parasites immediately after infection of its target cell (Anderson and Remington, 1974; McLeod *et al.*, 1980). In these in vitro systems, however, the parasite rapidly escapes the cytotoxic efforts of the activated macrophage, and begins to replicate even in the face of lymphokine treatment. These observations indicate that those mechanisms that effectively prevent parasite replication in immunologically normal, chronically infected hosts are fundamentally different from the mechanisms that underlie the transient cytotoxic phenomena observed in present in vitro systems. Further work to investigate the means by which cellular immune mechanisms prevent excystment and replication of *Toxoplasma* in mononuclear phagocytes and non-phagocytic cells will be necessary to elucidate this problem.

Two categories of amoebae cause disease in humans. The enteric pathogen, *Entamoeba histolytica*, primarily coexists with human hosts as an asymptomatic infection. Trophozoites live in the lumen of the large intestine, encyst there, and are passed in the stool as either trophozoites or cysts to infect other humans. Occasionally, *Entamoeba* may invade the bowel wall and cause amoebic dysentery; from there they travel through the portal system to the liver and cause amoebic liver abscess. Free-living amoebae, *Naegleria* and *Acanthamoeba*, cause amoebic meningoencephalitis. These organisms inhabit warm freshwater lakes. Amoebae penetrate the nasal mucosa of swimmers, infect the paranasal sinuses, and

invade the brain. The resulting meningoencephalitis is fatal despite antiamoebic chemotherapy. Acanthamoebae may also cause destructive ocular infection in wearers of contact lenses.

Amoebae pose an interesting challenge to immunocytes. Since the invader is itself phagocytic and possesses cytotoxins and digestive enzymes, it can ingest and destroy the cells that would eliminate it. Early studies, which investigated the antiamoebic role of antibody, complement and phagocytic cells, have failed to outline conclusively the crucial components of host defence against either free-living or enteric amoebae (Trissl, 1982). Recent investigations, however, suggest that lymphocytes and macrophages are the major protective effectors (Salata *et al.*, 1986; Salata *et al.*, 1985; Ghadirian and Meirovitch, 1981; Cleary and Marciano-Cabral, 1986a, b).

Pneumocystis carinii is among the most fascinating and enigmatic of protozoa that cause significant infection of extraluminal tissues (Walzer *et al.*, 1980). Asymptomatic pulmonary infection is common, but disease almost never occurs in immunologically normal individuals. Circumstances associated with T lymphocyte defects are generally associated with infection. Thus, patients who have lymphoid malignancies, who have received corticosteroids or cytotoxic chemotherapy, or who have congenital or acquired immunodeficiency syndromes, especially AIDS, are peculiarly susceptible to *Pneumocystis* pneumonia. Indeed, *Pneumocystis* pneumonia is the most significant infectious disease associated with AIDS. The disease may result from reactivation of long-standing latent infection or from recent acquisition of the organism. Despite the clear association of susceptibility to disease with impairment of cell-mediated immunity, the immunological mechanisms that control the parasite under most circumstances are unknown. In contrast to many organisms controlled by non-specific cellular immune processes, *Pneumocystis* is found exclusively extracellularly in alveolar fluid. Although the cellular response of immunodeficient patients to the organism is characterised by infiltration of monocytes and lymphocytes, the precise role of these cells in the destruction of the parasite in normal hosts is unknown. Alveolar macrophages cultured for less than two days in vitro fail to ingest *Pneumocystis* in the absence of specific antibody. In the presence of specific antiserum, however, macrophages ingest and kill the parasites (von Behren and Pesanti, 1978; Masur and Jones, 1978). Lack of a suitable animal model and inability to cultivate the organism axenically have hampered efforts to characterise further interactions of *Pneumocystis* with immune cells or their products. Elucidation of the factors that inhibit extracellular replication or enhance ingestion and

destruction of this parasite will require considerable ingenuity, but should prove to be an engrossing area for study.

Other protozoal infections are caused by luminal parasites. Of these, *Giardia lamblia, Crytosporidium* and *Trichomonas vaginalis* are the most significant. Despite their prevalence as human pathogens, they have received little attention from cellular immunologists. Evidence suggests, however, that co-operation between T cells and macrophages is important in defence against *Giardia*. Resistance to murine giardiasis requires participation of thymus-dependent cells; monocytes ingest and destroy the organisms (Roberts-Thompson and Mitchell, 1978; Smith *et al.*, 1982; Stevens *et al.*, 1978). The details of cell-mediated resistance, however, are not yet known.

III. INTERACTIONS OF PARASITIC PROTOZOA WITH NON-SPECIFIC DEFENCES

Host defence can be conveniently analysed by layers. Parasites must first overcome mucosal or integumentary barriers. They must resist soluble cytotoxic factors in extracellular fluid, and must avoid destruction by phagocytes or other killer cells. Obligate intracellular parasites must also avoid further obstacles to replication in their target cells. The course of infection with *Leishmania* illustrates the interplay of host defences and parasite avoidance mechanisms that result in disease, resistance to disease, or cure.

The skin is the first defensive layer encountered by *Leishmania*, which must eventually reach an intracellular environment within macrophages to survive. Sandflies bypass this defence by injecting the promastigote form of the parasite subepidermally. In the inflammatory environment of a bite, the parasite is exposed to serum-derived mediators of inflammation and components of the complement system. Promastigotes of *L. donovani* are killed by complement. Killing is mediated primarily by the classical pathway via absorbable factors, presumably cross-reacting antibody (Pearson and Steigbigel, 1981). The alternative complement pathway also participates in promastigote killing, without requirement for participation of antibody (Mosser and Edelson, 1984). Promastigotes grown to maximum cell concentration in vitro are more resistant to complement-mediated lysis than promastigotes in a logarithmic phase of growth (Franke *et al.*, 1985). These maturational or differentiative changes in parasite susceptibility to complement-mediated lysis may explain how sufficient promastigotes survive to infect host cells at the site of inoculation. This notion is supported by infectivity

studies: stationary-phase promastigotes not only resist lysis by complement, but also infect animal hosts more readily than do log-phase parasites (Gianini, 1974; Sacks and Perkins, 1984). Even after promastigotes have infected macrophages and transformed into amastigotes, serum factors may play a further role in host defence. To infect additional macrophages, amastigotes must be released extracellularly. There, like promastigotes, they are exposed to complement-rich inflammatory extracellular fluid (Ridley and Ridley, 1984). Amastigotes are killed by complement via the alternative pathway (Hoover *et al.*, 1984). Destruction of amastigotes does not require participation of antibody: serum adsorbed extensively with amastigotes remains fully cytotoxic. Indeed, *Leishmania major* amastigotes are extraordinarily sensitive to complement-mediated lysis: unlike most other targets, they are readily killed by serum deficient in components of the complement membrane attack complex (Hoover *et al.*, 1985a). These and other data suggest that complement-mediated defence may be important in control of the early stages of *Leishmania* infection. *Leishmania major*, which causes cutaneous leishmaniasis, is at least five-fold more sensitive to serum-mediated cytotoxicity than *L. donovani*, which causes disseminated, visceral disease (Hoover *et al.*, 1984). The importance of this phenomenon depends upon the mechanism by which *Leishmania* disseminate from the initial focus to distant sites of infection. Parasites may be protected from complement if they circulate within monocytes and macrophages, as described in patients with established visceral disease (Chulay *et al.*, 1985). If they travel extracellularly early in infection, however, they may be exposed to complement-mediated cytotoxicity. Complement may then preferentially destroy *L. major* so that visceral infection caused by that species dose not occur. In contrast, the relative resistance of *L. donovani* to complement-mediated cytotoxicity may favor its dissemination. Non-specific complement-mediated cytotoxicity is buttressed later in infection by specific antibody. Antibody enhances complement-mediated killing of *L. donovani* at least five-fold (Hoover *et al.*, 1985b). Specific humoral immunity does not, however, appear to play a role in recovery from leishmanial infection: visceral disease progresses despite high titres of antibody (Rezai *et al.*, 1978), and adoptive transfer of serum does not prevent infection of non-immune animals (Turk and Bryceson, 1971). Not only *Leishmania*, but also other flagellates are susceptible to antibody-independent complement-mediated killing. African trypanosomes and *Giardia lamblia*, for example, are killed by action of the alternative complement pathway (Flemmings and Diggs, 1978; Ferrante and Allison, 1983; Hill *et al.*, 1984).

Fundamental to establishment of intracellular infection is parasite entry into the host cell. Conceptually, entry may occur by effort of the parasite, of the host cell, or both. Susceptibility to infection and, conversely, resistance to infection, then may depend in part on the efficiency with which infection of individual cells occurs. For *Leishmania*, which replicate only in macrophages in the mammalian host, intracellular infection may require participation of both macrophage and parasite. Infection begins with parasite attachment to macrophages. Promastigotes preferentially attach by the flagellum, with a limited number of discrete contact points along the body of the organism (Chang, 1979; Pearson *et al.*, 1983a). Attachment is not a random phenomenon, but requires interaction of parasite membrane structures to specific receptor sites on macrophages (Chang, 1981). Promastigotes bind to both the mannose/fucose receptor and the CR3 receptor for C3bi (Blackwell, 1985). For effective internalisation, the promastigote must bind both receptors simultaneously (Blackwell *et al.*, 1985). Attachment of both promastigotes and amastigotes requires effort on the part of the parasite: it is inhibited by pre-treatment of parasites with cytochalasin B, which interferes with monofilament function (Aikawa, 1982; Wyler, 1982). This observation suggests that binding requires active maintenance by the parasite of some specific membrane structure. Cytochalasin treatment of macrophages, in contrast, prevents internalisation but not binding (Wyler, 1982). Other inhibitors of phagocytosis also inhibit internalisation of parasites. In sum, these findings indicate that leishmanial infection of macrophages is a co-operative activity initiated by the parasite but finally accomplished by the host cell (Silverstein, 1977). In effect, the parasite subverts macrophage defences to bring itself to an intracellular environment suitable for its replication.

To fully realise their goal of intracellular infection, parasites not only must bind to and enter the host cell, but must arrive at their destination alive. To do so, they must overcome the potent cytotoxicity of macrophage oxygen products. For *Leishmania*, the survival mechanisms are not apparent. *Leishmania* promastigotes are deficient in catalase and glutathione peroxidase, which scavenge H_2O_2 (Murray, 1981). Because of this deficiency, promastigotes are readily killed by H_2O_2. They do, however, resist destruction by other potentially toxic oxygen products (O_2^-, $OH\cdot$, and $^1O_2$) (Murray, 1981; Reiner and Kazura, 1983). Peroxide-mediated killing is markedly enhanced by the addition of lactoperoxidase and halide. Promastigotes of *L. major* are three-fold more sensitive than those of *L. donovani* to H_2O_2 (Murray, 1981). Not only are promastigotes extremely sensitive to H_2O_2, but they also induce its generation by mononuclear phagocytes upon association with the host-cell membrane

(Pearson *et al.*, 1983b). As a consequence of these phenomena, at least 80 per cent of promastigotes are destroyed when incubated with monocytes or macrophages cultured in vitro (Pearson *et al.*, 1983b; Murray, 1981). Given this great susceptibility to oxygen-mediated destruction by macrophages from non-immune animals, it is surprising that infection occurs at all! Other, unknown mechanisms must operate in vivo to prevent parasite destruction at the site of a bite. Other protozoans, for example, the trypomastigote and epimastigote forms of *T. cruzi*, are also highly susceptible to H_2O_2 (Nathan *et al.*, 1979). Nevertheless, *T. cruzi* also establishes intracellular infection of myocytes in vivo and mononuclear phagocytes in vitro (Nogueira and Cohn, 1976).

Other intracellular parasites do not share this marked susceptibility to oxygen-mediated cytotoxicity. *Leishmania* amastigotes, for example, are seven times as resistant as promastigotes to the lethal effects of H_2O_2, and survive macrophage contact to replicate intracellularly (Pearson *et al.*, 1983b). Similarly, *Toxoplasma* tachyzoites, which are endowed with plentiful scavengers (superoxide dismutase, catalase and glutathione peroxidase) of oxygen intermediates, resist H_2O_2 and O_2^- mediated killing (Murray and Cohn, 1979; Murray *et al.*, 1980). Moreover, *Toxoplasma* tachyzoites do not trigger the macrophage oxidative burst during ingestion (Wilson *et al.*, 1980).

The fate of intraphagocytic parasites depends not only on their susceptibility to oxygen-mediated cytotoxicity, but also on their ability to evade other potentially destructive macrophage products. For example, *Toxoplasma*, like *Leishmania*, replicates intracellularly in macrophages cultured in vitro. *Toxoplasma*, like *Leishmania*, enters the macrophage via phagocytosis (Nichols and O'Connor, 1981). The subsequent fate of these two intracellular organisms, however, is quite different. *Toxoplasma* inhibits fusion of parasitophorous vesicles and lysosomes in macrophages of non-immune animals (Jones and Hirsch, 1972). In the phagosome, protected from hostile lysosomal enzymes, tachyzoites replicate and eventually disrupt the host cell. Lysosome–phagosome fusion occurs normally if dead parasites are ingested, if viable parasites are ingested by macrophages from immune animals, or if immune serum is present during ingestion (Jones *et al.*, 1975). Under these conditions, viable tachyzoites are destroyed by intracellular microbicidal mechanisms. The mechanisms by which *Toxoplasma* prevents phagolysosomal fusion are not known. Similarly, the molecular basis for restoration of normal fusion by antibody or immune macrophages has not been elucidated. In contrast to *Toxoplasma*, *Leishmania* does not inhibit fusion of lysosomes and phagosomes, but replicates in the phagolysosome of macrophages from non-immune animals (Alexander and Vickerman, 1975; Chang and Dwyer, 1978). *Trypanosoma cruzi* also does not

inhibit phagolysosomal fusion, but escapes from the parasitophorous vacuole to replicate in the cytoplasm (Nogueira and Cohn, 1976).

The fate of intracellular *Leishmania* depends on the state of macrophage microbicidal capability. In general, the events that lead to macrophage activation for leishmanicidal activity parallel those elucidated by Mackaness (1962) in his pioneering work on resistance to infection with *Listeria monocytogenes*. Thus, *Leishmania* infect and replicate in resident macrophages harvested from the peritoneal cavity of non-immune, susceptible animals (Chang and Dwyer, 1978; Nacy and Diggs, 1981). In contrast, macrophages from immune animals previously infected with *Leishmania* destroy the parasites (Miller and Twohy, 1969). Macrophage cytotoxicity in cultures of cells from immune animals is related to the number of immune lymphocytes present with macrophages in peritoneal cell cultures (Mauel *et al.*, 1978). Induction of leishmanicidal activity in infected macrophages, however, is not entirely antigen-specific: lymphocytes from *Toxoplasma*-infected animals, for example, can substitute for lymphocytes from *Leishmania*-infected animals if *Toxoplasma* antigen is present in the culture. Conversely, lymphocytes from *Leishmania*-infected animals induce macrophage cytotoxicity for *Listeria* if *Leishmania* antigen is present in the culture (Behin *et al.*, 1975). These findings indicate that lymphocytes sensitive to one pathogen induce macrophage cytotoxicity for other, unrelated pathogens. This immunologically non-specific induction of cytotoxicity is mediated by lymphokines, factors secreted by stimulated lymphocytes that alter macrophage function. In a manner analogous to activation of complement or blood-clotting systems, lymphokine-induced macrophage activation involves participation of a number of molecules in a definite series of reactions. This process has been extensively analysed in C3H/HeN mouse peritoneal macrophages cultured in vitro as a non-adherent cell pellet. The amastigote form of *L. major* readily infects these cells, and replicates 5–10-fold over 72 hours of culture (Nacy and Diggs, 1981). In contrast, macrophages treated in vitro with lymphokines (supernatant fluids from cultures of PPD-stimulated spleen lymphocytes from BCG-immune mice or of concanavalin-A-stimulated cells from non-immune mice) develop potent antileishmanial activity (Nacy *et al.*, 1981; Pappas and Nacy, 1983). This activity has two distinct components. The first component, resistance to infection, occurs in macrophages treated with lymphokines for at least four hours prior to exposure to amastigotes. Lymphokine pre-treatment reduces leishmanial infection of macrophages by 30–50 per cent compared with untreated cells (Oster and Nacy, 1984). Following the pre-treatment period, macrophages retain their resistance to infection with amastigotes for at least 24 hours. The mechanistic explanation of this phenomenon is not yet known; it is not

due to generalised depression of phagocytic activity, for treated macrophages ingest latex beads and sheep erythrocytes in a normal fashion (Nacy *et al.*, 1981). It may reflect extracellular killing of parasites, perhaps through the oxidant-mediated mechanisms discussed later. Alternatively, it may be due to alterations in specific receptor-mediated ingestion of parasites or to alterations in other, as yet undefined, mechanisms of parasite entry. Whatever its mechanism, resistance to infection is an intriguingly unique response to an obligate intracellular organism: denied its essential environment, the parasite may fall prey to humoral defence mechanisms in the extracellular milieu.

Lymphokines also induce another, far more potent, antileishmanial activity in resident macrophages. This activity, intracellular killing, occurs in macrophages treated with lymphokines after infection with amastigotes. After culture for 72 hours, the percentage of infected cells in lymphokine-treated cultures is reduced by 80–100 per cent compared to the percentage of infected cells in medium-treated cultures. Leishmanicidal activity is induced in macrophages by a number of lymphocyte-derived factors (Meltzer *et al.*, 1986). Most of the antileishmanial macrophage-activating activity in crude lymphokine preparations is due to gamma interferon (IFN-γ). As little as 5 IU/mL of IFN-γ induces maximal microbicidal activity in murine macrophages (Nacy *et al.*, 1985). Non-glycosylated IFN-γ produced by recombinant DNA technology and glycosylated forms of the molecule secreted by T cell hybridomas have equivalent activity. Most antileishmanial activity in crude lymphokine is removed by treatment with anti-IFN-γ. Anti-IFN-γ is effective both when added as soluble antibody to the culture fluids, and when affixed to a solid support matrix to remove IFN-γ from solution. Approximately one-third of antileishmanial activity remains, however, when lymphokine preparations are treated with anti-IFN (Nacy *et al.*, 1985). Thus, non-IFN molecules induce murine leishmanicidal activity. The scope of functions mediated by these agents is not yet known. It is possible that multiple factors are required to achieve maximal cytotoxicity in vivo. Alternatively, different factors may be secreted preferentially to fine-tune macrophage responses at different phases of the host response to infection. This concept, that multiple lymphokine factors participate in the regulation of macrophage leishmanicidal activity, leads to the prediction that inhibitory factors should also exist. In fact, at least one such lymphokine has been described in the leishmanicidal system. This factor, secreted by the EL-4 mouse thymoma cell line, abrogates macrophage microbicidal activity in response to IFN or non-IFN activation factors, but does not by itself enhance amastigote replication

in macrophages (Nacy, 1984). Although this molecule has so far proven difficult to characterise and purify by biochemical means, it has provided intriguing information on mechanisms of control of the antileishmanial activity induced by IFN and non-IFN macrophage activation factors. Purification of non-IFN macrophage activation factors will provide important reagents for further analysis of interactions between *Leishmania* and macrophages.

Although the cytotoxic response of lymphokine-treated macrophages is dramatic, it requires rather precise conditions for optimal effect. Timing, for example, is crucial. Macrophage responsiveness for lymphokine-induced cytotoxicity decays rapidly with time of culture. After culture for eight hours prior to lymphokine treatment, macrophages develop only two-thirds maximal leishmanicidal activity. They are completely unresponsive if cultured for 24 hours prior to treatment (Oster and Nacy, 1984). These findings indicate that some as yet undefined factors present in vivo but absent from in vitro culture systems maintain the macrophage in a receptive state for the action of macrophage activation factors.

Certain aspects of this 'priming' phenomenon can be recapitulated by experiments with brief pulses of lymphokine in vitro (Fig. 22.1) (Nacy *et al.*, 1984). Treatment of macrophages with high concentrations of lymphokine for 4–8 hours elicits microbicidal activity at 72 hours equivalent to that induced by treatment with lymphokine throughout the entire culture period. Macrophages pulsed with lymphokine for less than four hours fail to develop microbicidal activity. During this crucial four-hour time period of continuous exposure to low or high concentrations of lymphokine, the resting macrophage is 'primed': it becomes receptive to 'triggering' by a short pulse (15 minutes) of high-dose lymphokine or immunologically non-specific stimuli such as lipopolysaccharide (LPS) or certain plant lectins. The requirement for several hours of priming is crucial. Administration of fully microbicidal concentrations of lymphokine for 15–60 minutes at the beginning of the experiment will not prime macrophages to respond to a subsequent 15 minute lymphokine pulse. The order of treatment with priming and triggering agents is also important. Agents such as LPS, though capable of triggering the microbicidal response, do not prime the macrophage for triggering by LPS or lymphokine. These findings suggest that intervention by lymphokine may provide specificity to the microbicidal response. Further specificity is provided by distinct pathways for non-IFN lymphokine and IFN during the priming and triggering sequence. Non-IFN lymphokine does not prime macrophages to respond to IFN, and IFN does not prime

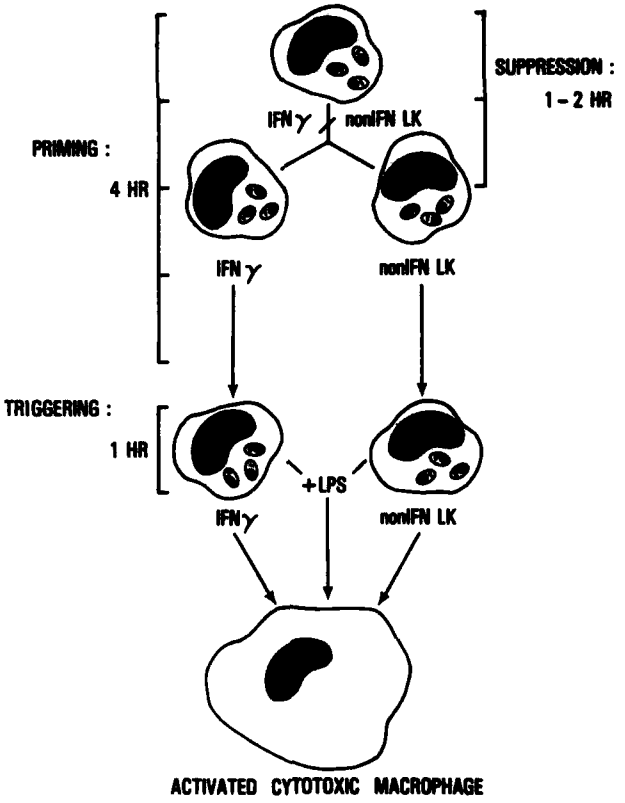

Fig. 22.1: Sequential steps in the activation of macrophages for intracellular killing. IFN = interferon; LK = lymphokine; LPS = lipopolysaccharide

macrophages to respond to non-IFN lymphokine. Interestingly, the suppressor lymphokine produced by EL-4 cells inhibits priming by both IFN and non-IFN lymphokine (Nacy, 1984).

One should emphasise that these interpretations depend heavily on the nature of the in vitro system used to analyse interactions among *Leishmania*, macrophages, and mediators. This caveat is clearly demonstrated in findings with human monocytes. Freshly harvested human monocytes cultured as a non-adherent cell pellet, as in the preceding experiments with mouse peritoneal macrophages, respond to IFN and to at least one other non-IFN lymphokine to kill *L. donovani* (Hoover *et al.*, 1986). Expression of microbicidal activity requires only a brief pulse with lymphokine or IFN immediately before or within a few hours of infection in vitro (Hoover *et al.*, 1985c). In another system, developed by

Murray and others, adherent monocytes are cultured for 7–10 days prior to lymphokine treatment (Murray and Cartelli, 1983). During this period of culture, monocyte production of oxidative products declines dramatically (Nakagawara *et al.*, 1981). It can, however, be boosted back to levels of freshly harvested cells by treatment with lymphokine. Lymphokine must be administered for three days prior to infection to induce optimum oxidative and microbicidal effect (Murray and Cartelli, 1983). Oxidative killing may occur primarily during parasite entry into the cell. It is also possible that killing occurs intracellularly by oxidative and non-oxidative mechanisms. In contrast to the non-adherent cell system, microbicidal activity in the adherent cell system occurs only in response to IFN. Non-IFN molecules are without effect (Murray *et al.*, 1983b; Nathan *et al.*, 1984).

Lymphokine-stimulated macrophages also kill *Toxoplasma gondii* and *Trypanosoma cruzi*. A number of studies have indicated that destruction of these two organisms is associated with increased secretion of oxidative products by lymphokine-stimulated macrophages (Murray *et al.*, 1979; Murray and Cohn, 1980; Murray *et al.*, 1980; Locksley *et al.*, 1982; Wilson *et al.*, 1980). It is not yet known which oxygen products mediate the cytotoxic event. *T. gondii* is more resistant than *T. cruzi* to H_2O_2, perhaps due to its higher concentration of catalase (Nathan *et al.*, 1979; Murray and Cohn, 1979). For *T. gondii*, metabolites of H_2O_2 such as $OH\cdot$ or $^1O_2$ may be more effective agents of cytotoxicity (Murray and Cohn, 1979). In contrast to these findings, other observations indicate that antitoxoplasma activity by lymphokine-stimulated human monocytes does not require production of reactive oxygen metabolites (Wilson and Haas, 1984). These data suggest that non-oxygen-mediated microbicidal mechanisms may be more important than oxidative mechanisms for destruction of *Toxoplasma*. One should note that *T. cruzi* and *T. gondii*, unlike *Leishmania*, replicate in cells other than macrophages. Indeed, the majority of parasites in infected animals may reside in non-phagocytes. Interestingly, IFN inhibits replication of *Toxoplasma* in human fibroblasts cultured in vitro. This antitoxoplasma effect is associated with enhanced degradation of tryptophan by host cells (Pfefferkorn and Guyre, 1983). The effects of lymphokine and other immune mediators on non-phagocytic cells that support parasite growth will be an intriguing area of investigation for the future (Murray *et al.*, 1983a).

Expression of lymphokine-induced microbicidal activity depends not only on several signals delivered to macrophages in a precise order, it also depends on the state of maturation and/or differentiation of the macrophage. Inflammatory peritoneal macrophages that have recently emigrated from the blood express less microbicidal activity in response

to lymphokine than do mature, resident cells (Hoover and Nacy, 1984). Murine blood monocytes respond even more poorly than inflammatory macrophages for leishmanicidal activity. These observations suggest that macrophages that arrive early at a site of leishmanial infection may be readily infected with amastigotes, but may be unable to kill them. Recent data lend support to this hypothesis. Adoptive transfer of T cells that mediate delayed-type hypersensitivity to *Leishmania* causes increased lesion size in normally leishmania-resistant animals (Titus *et al.*, 1984). These large lesions contain more macrophages that are more heavily infected than cells in lesions of control animals. Chemotaxis of large numbers of immature macrophages that are incapable of leishmanicidal activity might be responsible for this paradoxically detrimental effect of sensitised lymphocytes.

Other adoptive transfer experiments have demonstrated remarkably complex interactions between suppressive and enhancing cell populations in control of leishmanial infection. A number of investigators have analysed infection of CBA or BALB/c mice with *L. major* (Preston and Dumonde, 1976; Howard *et al.*, 1980; Scott and Farrell, 1981; Mitchell *et al.*, 1981). This species causes localised cutaneous disease in humans, but results in extensive local necrotic lesions, disseminated infection, and eventually death in BALB/c mice. Susceptibility to fatal infection can be abrogated by sublethal irradiation of mice 10 days prior to infection. These irradiated mice heal their infection in a manner similar to other, resistant strains of mice (Howard *et al.*, 1981). Interestingly, susceptibility can be conferred on irradiated BALB/c mice by adoptive transfer of T cells from non-irradiated susceptible animals (Howard *et al.*, 1982). These suppressor T cells paradoxically express a Lyt 1^+2^- IJ^- phenotype. The mechanism by which suppressor cells enhance susceptibility is not yet known. Reduced lymphokine production by lymph-node cells from infected, susceptible animals may be one component of impaired host response (Sadick *et al.*, 1986).

Other cell-mediated suppressive influences have also been described in severe leishmanial infections. For example, monocytes suppress antigen-induced blastogenesis of lymphocytes from the blood of patients with diffuse cutaneous leishmaniasis (Petersen *et al.*, 1984). Similarly, adherent cells in spleens of BALB/c mice infected with *L. major* inhibit spleen lymphocyte proliferation in response to mitogens or leishmanial antigens (Scott and Farrell, 1981). Other reports indicate that reduced lymphocyte responses to mitogens or antigens may not require the action of suppressor cells (Carvalho *et al.*, 1981; Castes *et al.*, 1983). Blood mononuclear cells from patients with untreated American visceral leishmaniasis showed impaired proliferation to antigens, but not to non-

specific mitogens (Carvalho *et al.*, 1981). With successful therapy, however, antigen-induced proliferation was restored. Monocyte-enriched cell fractions did not mediate suppression in these studies. Antigen-specific lymphocyte proliferation and IFN production in vitro may also be reduced before treatment of extensive cutaneous leishmaniasis, but return to normal with treatment (Murray *et al.*, 1984). Studies in *L. donovani*-infected mice reached similar conclusions: reduced interleukin 2 (IL-2) production by lymphocytes of infected animals was not mediated by macrophages or suppressive factors in supernatant fluids of cell cultures (Reiner and Finke, 1983). These findings indicate that during the course of leishmaniasis, numerous influences regulate production of lymphokines and lymphocyte proliferative responses. The interplay of these influences may result in prolongation of the disease state, but also terminates host response as infection is successfully resolved.

IV. IMMUNOTHERAPEUTIC APPROACHES

With the recognition that natural immunity inhibits progression of disease caused by a number of protozoa have come efforts to enhance resistance to infection by administration of 'non-specific' immunomodulating agents. Administration of BCG to BALB/c mice prior to infection with *L. donovani* reduced the number of organisms found in spleen or liver, but was not curative (Smrkovski and Larson, 1977). Similarly, administration of glucan, a polyglucose derivative from yeast cell walls, reduced the number of parasites in the livers and spleens of CF1 mice infected with *L. donovani* (Holbrook *et al.*, 1981). Glucan also reduced parasite numbers in hamsters infected with *L. donovani* (Cook *et al.*, 1982). Moreover, macrophages from these glucan-treated hamsters inhibited replication of *Leishmania*. The pathway by which glucan treatment induces antileishmanial activity in macrophages in vitro, and presumably in vivo, is unknown. Further study of non-specific potentiators of macrophage function for defence against intracellular organisms will be of great interest. Other non-specific factors produced by specifically sensitised immune cells have also been tested for therapeutic efficacy in vivo. Administration of liposome-encapsulated lymphokines, for example, reduced parasite burdens in *L. chagasi*-infected mice (Reed *et al.*, 1984). Survival of *Toxoplasma*-infected mice was also improved by treatment with IL-2 or IFN (Sharma *et al.*, 1985; McCabe *et al.*, 1984). In vitro studies of cells from IL-2-treated mice indicated no enhancement of macrophage antitoxoplasma activity, but demonstrated enhanced natural killer cell cytotoxicity against the parasite (Sharma *et al.*, 1985).

V. CONCLUSION

As this brief review indicates, interactions of protozoan parasites with natural immune mechanisms are exceedingly complex. As a consequence, we presently understand these events at a phenomenologic level, rather than at a molecular level. It is likely that, as our understanding deepens, we will perceive more specificity in the mechanisms that enhance or diminish antiprotozoal responses. Recognition of this specificity may provide tools for rational immunotherapy or immunoprophylaxis of disease caused by this fascinating and diverse group of organisms.

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